(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 January 2001 (25.01.2001)

PCT

(10) International Publication Number
WO 01/05434 A2

(51) International Patent Classification[7]: A61K 47/48

(21) International Application Number: PCT/US00/19803

(22) International Filing Date: 20 July 2000 (20.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/144,639 20 July 1999 (20.07.1999) US
09/619,069 19 July 2000 (19.07.2000) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/05434 A2

(54) Title: HYALURONIC ACID-PROTEIN CONJUGATES, PHARMACEUTICAL COMPOSITIONS AND RELATED METHODS

(57) Abstract: The present invention broadly relates to the field of protein modification and, more specifically, the attachment of low molecular weight, derivatized hyaluronic acid polymer to proteins including leptin and analogs thereof (the term "protein" as used herein is synonymous with "polypeptide" or "peptide" unless otherwise indicated). The hyaluronic acid-protein conjugates of the present invention exhibit longer sustained blood levels than formulations containing protein alone, thus providing an important advantage in the therapeutic setting.

HYALURONIC ACID-PROTEIN CONJUGATES, PHARMACEUTICAL COMPOSITIONS AND RELATED METHODS

BACKGROUND OF THE INVENTION

Due to recent advances in genetic and cell engineering technologies, proteins known to exhibit various pharmacological actions *in vivo* are capable of production in large amounts for pharmaceutical applications. Such proteins include erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), interferons (alpha, beta, gamma, consensus), tumor necrosis factor binding protein (TNFbp), interleukin-1 receptor antagonist (IL-1ra), brain-derived neurotrophic factor (BDNF), kerantinocyte growth factor (KGF), stem cell factor (SCF), megakaryocyte growth differentiation factor (MGDF), osteoprotegerin (OPG), glial cell line derived neurotrophic factor (GDNF), novel erythropoiesis stimulating protein (NESP) and obesity protein (OB protein). OB protein may also be referred to herein as leptin.

The availability of these therapeutic proteins has engendered advances in protein formulation and chemical modification. The goals of such modification include protein protection, increasing the stability, solubility, and half-life of the protein, as well as favorably affecting the *in vivo* distribution of the therapeutic protein and decreasing immunogenicity.

Chemical modification using water-soluble polymers has been extensively studied, and several polymer-protein conjugate formulations having improved properties have been reported. For example, U.S. Patent No. 5,824,784, discloses N-terminally monopegylated granulocyte colony stimulating factor ("G-CSF") and N-terminally monopegylated consensus interferon ("N-terminally monopegylated" denoting that

the protein moiety has attached to it a single polyethylene glycol moiety at the N-terminus) which demonstrate, *inter alia*, improved stability.

Other water-soluble polymers used include copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran.

Dextrans are polysaccharide polymers comprised of individual subunits of glucose predominantly linked by α1-6 linkages. The α1-6 linkage in dextran is quite resistant to the normal body carbohydrases and the polymer is slowly hydrolyzed, a property which is relied upon in the use of dextran as a blood volume expander. Unfortunately, large molecular weight dextrans, when administered in gram amounts, fail to produce adequate ultrafiltration and are reported to result in kidney vacuoles; *see, e.g.*, Diomi et al., *Annals of Surgery*, 172:813-24 (1970)(reporting kidney vacuolization in dogs); Maunsbach et al., *Laboratory Investigation*, 11:421-32 (1962)(reporting kidney vacuolization in rats); and Engberg, *Acta Chir. Scand.*, 142:172-80 (1976). Polyethylene glycol-protein conjugates, in particular instances, have also been associated with kidney vacuole induction; Bendele et al., *Toxicological Sciences*, 42:152-57 (1998). Although kidney vacuoles are not currently understood to be clinically relevant, in general, a pharmaceutical composition should be efficacious without causing unwarranted anatomical changes. Thus, large molecular weight dextrans and certain polyethylene glycol polymers may not be

generally applicable for chemical modification of all therapeutic proteins.

Another area where extensive studies have been directed involve chemical modification utilizing hyaluronic acid. Hyaluronic acid is a viscoelastic biomaterial composed of repeating disaccharide units of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcUA), and is a major component of the extracellular matrix. Hyaluronic acid often occurs naturally as the sodium salt, sodium hyaluronate, and hyaluronic acid/sodium hyaluronate preparations are generally referred to as "HA". Highly purified, high molecular weight hyaluronate is used in viscosurgery and frequently used in ophthalmic surgery.

U.S. Patent Nos. 5,874,417 and 5,616,568, and references cited therein, describe past efforts directed at chemical modification using hyaluronate, or functionalized derivatives thereof. More specifically, the patents describe various functionalized derivatives of hyaluronic acid and their use as a gel-forming component for the sustained delivery of small molecule drugs from biocompatible gels or hydrogels; and/or as an aid to the spreading of the co-injected small molecule drugs.

It is an object of the present invention to prepare low molecular weight, derivatized hyaluronic acid-protein conjugates, which exhibit longer sustained blood levels than formulations containing protein alone. The conjugates of the present invention are not biocompatible gels, hydrogels, or topical formulations and can be injected subcutaneously.

SUMMARY OF THE INVENTION

The present invention stems from the observation that low molecular weight hyaluronate

(10-80 kD) of any derivatization (1-100% of monomer modified) is useful as a biocompatible, biodegradable water-soluble polymer for conjugation to protein therapeutics. Unexpectedly, certain HA-protein conjugates surprisingly possess the desirable characteristics of improved efficacy and circulation time and possess other desired characteristics such as increased solubility and reduction in injection site reactions as compared to native protein.

The working examples set forth below demonstrate HA-protein conjugates which have the following characteristics: (a) improved efficacy over unmodified recombinant human protein; (b) an extended plasma circulation time over unmodified recombinant human protein; (c) improved aqueous solubility at physiologic pH; (d) mild or non-existent injection site reactions; and (e) improved *in vivo* distribution.

Therefore, in one aspect, the present invention provides HA-protein conjugates comprising at least one derivatized HA moiety attached to at least one protein moiety. A particularly preferred derivatized HA moiety is about 20kD, as exemplified in the Examples below.

In yet another aspect, the present invention relates to pharmaceutical compositions containing the present HA-protein conjugates in a pharmaceutically acceptable carrier.

The present invention also relates to methods of treatment of individuals using HA-protein conjugates.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides HA-protein conjugates having the advantages of improved efficacy and longer plasma circulation time. Additional

advantages of the HA-protein conjugates of the present invention include improved stability, solubility and *in vivo* distribution.

Hyaluronate is a useful biomedical polymer, which is biocompatible, non-immunogenic, and subject to natural degradation by enzymes in the body. Commercially available HA and salts, and their methods of preparation are well known. HA used in the practice of this invention include low molecular weight HA's having a molecular weight from about 10 kD to about 80 kD. A particularly preferred HA for ease in commercial manufacture of a pharmaceutical for human use is one having a molecular weight of about 20 kD.

The HA moieties of the present invention are also "derivatized" to improve various properties for conjugation to a protein moiety. Such derivatization involves, e.g., the addition of adipic dihydrazide to the HA, by route of activation with carbodiimide.

The derivatized HA is then "activated" to a form that contains maleimide groups for subsequent reaction with a biologically active agents free thiol group. This activation method generally utilizes crosslinkers such as sulfosuccinimidyl 4-[*p*-maleimidophenyl]-butyrate.

The HA-protein conjugates of the present invention may be characterized using well-known methods in the art such as SDS-PAGE analysis, mass spectrometry, RP-HPLC peptide mapping, GPC, GFC, light scattering and N-terminal sequence analysis.

As used herein, biologically active agents refers to recombinant or naturally occurring proteins, whether human or animal, useful for prophylactic, therapeutic or diagnostic application. The biologically active agent can be natural, synthetic, semi-synthetic or derivatives thereof. A wide range of biologically active agents are contemplated. These

include but are not limited to hormones, cytokines, hematopoietic factors, growth factors, antiobesity factors, trophic factors, anti-inflammatory factors, and enzymes (see also U.S. Patent No. 4,695,463 for additional examples of useful biologically active agents). One skilled in the art will readily be able to adapt a desired biologically active agent to the compositions of present invention which can also include small organic or organometallic compounds.

Such proteins would include but are not limited to granulocyte-colony stimulating factors (G-CSF's) (*see*, U.S. Patent Nos. 4,810,643, 4,999,291, 5,581,476, 5,582,823, and PCT Publication No. 94/17185, hereby incorporated by reference including drawings), interferons (*see*, U.S. Patent Nos. 5,372,808, 5,541,293 4,897,471, and 4,695,623 hereby incorporated by reference including drawings), interleukins (*see*, U.S. Patent No. 5,075,222, hereby incorporated by reference including drawings), erythropoietins (*see*, U.S. Patent Nos. 4,703,008, 5,441,868, 5,618,698 5,547,933, and 5,621,080 hereby incorporated by reference including drawings), stem cell factor (PCT Publication Nos. 91/05795, 92/17505 and 95/17206, hereby incorporated by reference including drawings), osteoprotegerin (PCT Publication No. 97/23614, hereby incorporated by reference including drawings), novel erythropoiesis stimulating protein (NESP) (PCT Publication No. 94/09257, hereby incorporated by reference including drawings) and leptin (OB protein).

Also included are those proteins as set forth above with amino acid substitutions which are "conservative" according to acidity, charge, hydrophobicity, polarity, size or any other characteristic known to those skilled in the art. These are set forth in Table 1, below. *See generally*, Creighton, *Proteins*, *passim* (W.H. Freeman and Company,

N.Y., 1984); Ford et al., *Protein Expression and Purification*, 2:95-107 (1991), which are herein incorporated by reference.

Table 1

Conservative Amino Acid Substitutions

Basic:	arginine lysine histidine
Acidic:	glutamic acid aspartic acid
Polar:	glutamine asparagine
Hydrophobic:	leucine isoleucine valine
Aromatic:	phenylalanine tryptophan tyrosine
Small:	glycine alanine serine threonine methionine

In addition, biologically active agents can also include but are not limited to insulin, gastrin, prolactin, adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), human chorionic gonadotropin (HCG), motilin, interferons (alpha, beta, gamma), interleukins (IL-1 to IL-12), tumor necrosis factor (TNF), tumor necrosis factor-binding protein (TNF-bp), brain derived neurotrophic factor (BDNF),

glial derived neurotrophic factor (GDNF), neurotrophic factor 3 (NT3), fibroblast growth factors (FGF), neurotrophic growth factor (NGF), insulin-like growth factors (IGFs), macrophage colony stimulating factor (M-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), megakaryocyte derived growth factor (MGDF), keratinocyte growth factor (KGF), thrombopoietin, platelet-derived growth factor (PGDF), colony simulating growth factors (CSFs), bone morphogenic protein (BMP), superoxide dismutase (SOD), tissue plasminogen activator (TPA), urokinase, somatotropins, streptokinase and kallikrein. The term proteins, as used herein, includes peptides, polypeptides, consensus molecules, analogs, derivatives or combinations thereof.

In yet another aspect of the present invention, provided are pharmaceutical compositions of the present HA-protein conjugates and methods of treatment using such pharmaceutical compositions for therapeutic uses. Such pharmaceutical compositions may be for administration by bolus injection or by infusion (e.g., intravenous or subcutaneous), or for oral, pulmonary, nasal, transdermal or other forms of administration.

In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of HA-protein conjugates of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such pharmaceutical compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking

substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. *See, e.g.*, PCT WO 96/29989, herein incorporated by reference. *See, e.g.*, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form. Implantable sustained release formulations are also contemplated, as are transdermal formulations.

Contemplated are oral formulations, as described in PCT WO 95/21629, herein incorporated by reference in its entirety. This PCT publication describes oral delivery of chemically modified proteins, including proteins modified by amylose moieties. The compositions and methods disclosed therein are applicable here to prepare oral delivery formulations of the present HA-protein conjugates.

Pulmonary delivery of the present HA-protein conjugates is also contemplated, and compositions and methods disclosed in PCT WO 94/20069 are useful for the preparation and use of the present HA-protein conjugates. WO 94/20069, which discloses the pulmonary delivery of chemically-modified G-CSF, is herein incorporated by reference.

Therapeutic uses of the compositions of the present invention depend on the biologically active agent used. One skilled in the art will readily be able to adapt a desired biologically active agent to the present invention for its intended therapeutic uses. Therapeutic uses for such agents are set forth in greater detail in the following publications hereby incorporated by reference including drawings. Therapeutic uses include but are not limited to uses

for proteins like granulocyte-colony stimulating factors (*see*, U.S. Patent Nos. 4,999,291, 5,581,476, 5,582,823, 4,810,643 and PCT Publication No. 94/17185, hereby incorporated by reference including drawings), interferons (*see*, U.S. Patent Nos. 5,372,808, 5,541,293, hereby incorporated by reference including drawings), interleukins (*see*, U.S. Patent No. 5,075,222, hereby incorporated by reference including drawings), erythropoietins (*see*, U.S. Patent Nos. 4,703,008, 5,441,868, 5,618,698 5,547,933, and 5,621,080 hereby incorporated by reference including drawings), stem cell factor (PCT Publication Nos. 91/05795, 92/17505 and 95/17206, hereby incorporated by reference including drawings), OB protein (*see* PCT publication Nos. 96/40912, 96/05309, 97/00128, 97/01010 and 97/06816 hereby incorporated by reference including figures), novel erythropoiesis stimulating protein (PCT Publication No. 94/09257, hereby incorporated by reference including drawings), and small molecule drugs. In addition, the present compositions may also be used for manufacture of one or more medicaments for treatment or amelioration of the conditions the biologically active agent is intended to treat.

One skilled in the art will be able to ascertain effective dosages by administration and observing the desired therapeutic effect. The effective dosages may be determined using diagnostic tools over time. For example, a diagnostic for measuring the amount of protein in the blood (or plasma or serum) may first be used to determine endogenous levels of the protein. Such diagnostic tool may be in the form of an antibody assay, such as an antibody sandwich assay. The amount of endogenous protein is quantified initially, and a baseline is determined. The therapeutic dosages are determined as the

quantification of endogenous and exogenous protein moiety (that is, protein, analog or derivative found within the body, either self-produced or administered) is continued over the course of therapy. The dosages may therefore vary over the course of therapy, with, for example, a relatively high dosage being used initially, until therapeutic benefit is seen, and lower dosages used to maintain the therapeutic benefits.

Selected HA-protein conjugates of the present invention may be isolated from HA-protein conjugate mixtures using well-known methods in the art for purifying HA and/or protein.

Example 1

This example describes the preparation of the derivatized, low molecular weight hyaluronate used in the preparation of the HA-protein conjugates.

To 200 mL of stirred hyaluronidase buffer (23 mM sodium phosphate, 140 mM NaCl, pH 6.4) in a one liter beaker was added 4.00 gm native sodium hyaluronate. The native sodium hyaluronate was added in approximately 0.8 gm portions and alternated with 30 mL portions of extra buffer, resulting in a well dispersed, stirring mixture (total volume ~ 380 mL). Vigorous stirring was continued for at least 4 hours at 37°C to fully homogenize and warm.

Enzyme was then added as a freshly prepared solution in buffer (2 aliquots of 4.5 mL, 6.2 mg net enzyme, Sigma, 320 units/mg, Type 1-S), and well incorporated by physical swirling of the entire reaction vessel for 3 to 5 minutes. Within 1 hour the rate of stirring was slowed to better suit the drop in solution viscosity. Stirring was continued at 37°C for another 17 hours.

The solution was moved to a cold box (4°C) and stirred slowly for 6 hours. Product was precipitated using 99% isopropanol that was pre-chilled to between -10°C and 0°C in a 450 mL alcohol + 48 mL aqueous ratio. Pellets were obtained by centrifugation at 2,440 x g, 6°C, for 8 minutes. Due to volume constraints, supernatants were decanted, fresh cold isopropanol was added (450 mL) followed by HA aqueous (48 mL), and in this manner a second precipitation/pelleting was performed over top of the initial pellet. Pellets were air dried overnight, dissolved in water to ~40 mg/mL, placed into dialysis tubing (3.5 K Mw cutoff) and dialysed against water. After 2 days and 4 baths of 20x volume at 4°C, samples were removed, 0.45 µm filtered, frozen, and lyophilized. This material was designated HA9 and had M.W. of ~18,600.

To prepare a highly derivatized ("60x"), low molecular weight hyaluronate, 1065 mg of HA9 was dissolved in 100 mL of 100 mM Bis-Tris buffer, pH 4 at 21°C. To this solution was added 6.15 g ADH (35 mmoles). After dissolution, the pH was dropped to 4.7 by the addition of 2 mL of 1.0 N HCl. The addition of EDCI, (1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, 243 mg, 1.24 mmoles) caused an increase in pH over a 25 minute stirring period. The pH was then reduced from 4.83 to 4.70 with the addition of 0.50 mL of 1.0 N HCl. After 60 minutes more reaction, the pH again was adjusted with HCl, from 4.77 to 4.70. 5 hours total reaction time was concluded with the addition of sodium azide (25 mg for 3 minutes) followed by dialysis in 3.5 kD Mw cutoff tubing, against water, at 4°C (3.8 L/bath x 4 baths over 2 days). Samples were then 0.45 µm filtered, frozen and set to

lyophilize. The final preparation was designated DM2-66.

Alternatively, one could prepare a low molecular weight hyaluronate having a lower level of derivatization ("50x"), herein referred to as DM2-63. This synthesis followed the protocol above with changes to the amounts of materials as described, (1145 mg of HA9, 1.554 g ADH, and 55 mg EDCI).

The above materials were assayed for hydrazide content by a modified ninhydrin assay, monitored at 414 nm, using adipic dihydrazide as standard. In brief, 19 mL of methanol was added to 6.5 mL of 4 N HOAc (pH 4). 90 mg hydrindantin and 508 mg ninhydrin monohydrate was added and the mixture shaken for 3 hours. After pelleting undissolved solids by centrifuge, 0.25 mL of the supernatant was reacted with 0.50 mL of aqueous sample for 30 minutes at 100°C. Brief cooling was followed by dilution with 0.75 mL of 50% ethanol/water, then measurement of absorbance at 414 nm.

Example 2

This example describes the activation of the derivatized hyaluronate of Example 1 and the subsequent conjugation of the activated HA moiety to various protein moieties to form a various protein conjugates.

130 mg of derivatized HA, DM2-66, prepared as described in Example 1 and containing an estimated 260 µmoles hydrazide was dissolved in 2.0 mL of 30 mM phosphate, 5 mM EDTA, pH 6.55 at 5°C. To half of this solution was added 0.50 mL water followed by 16 mg sulfo-SMPB (32 µmoles) at room temperature. The pH of the mixture was adjusted to 6.2, and stirred for 35 minutes before the addition of 60 µL DMSO, 60 µL

0.60 M dibasic sodium phosphate, and sufficient 1.0 M NaOH to yield pH 7.0. After 60 minutes, the small molecules were removed by passing the reaction through PD-10 columns equilibrated in the phosphate/EDTA buffer aforementioned. 1 mL of the 3.5 mL eluent was then reacted with 42 mg of prepared free-thiol (N-terminus) leptin (~ >5 μmoles maleimide plus 2.6 μmoles leptin thiol). After 4 days of reaction at 4°C, analysis by SDS-PAGE gel suggested free leptin still in abundance. The second half of the initially dissolved derivatized HA sample was then activated in a similar fashion, added to the reaction with protein, and this was tumbled for 5 days.

136 mg derivatized HA, DM2-66, prepared as described in Example 1 and containing an estimated 260 μmoles hydrazide was dissolved in 4.0 mL of phosphate buffer, pH 7 at 4°C. To this solution (now at 21°C) was added 31 mg sulfo-SMPB and 1 mL water. The mixture was stirred for 90 minutes before being 0.45 μm filtered and PD-10 buffer exchanged. This was then reacted with 19 mg of free-thiol osteoprotegerin (OPG). After 2 days of reaction at 4°C, the sample was pH adjusted from 6.3 to 6.9 with 0.10 N NaOH. After a total of 2 weeks reaction, 120 mg more activated HA-ADH was added, and the reaction allowed to run another 2 weeks.

130 mg derivatized HA, DM2-66, prepared as described in Example 1 and containing an estimated 260 μmoles hydrazide was dissolved in 2.0 mL of 30 mM phosphate, 5 mM EDTA, pH 6.55 at 5°C. To half of this solution was added 0.50 mL water followed by 16 mg sulfo-SMPB (32 μmoles) at room temperature. The pH of the mixture was adjusted to 6.2 and then stirred for 35 minutes before the addition of 60 μL DMSO, 60 μL

0.60 M dibasic sodium phosphate, and sufficient 1.0 M NaOH to yield pH 7.0. After 60 minutes, the small molecules were removed by passing the reaction through PD-10 columns equilibrated in the phosphate/EDTA buffer aforementioned. 2.5 mL of the 3.5 mL eluent was then reacted with 77 mg of IL-1ra in the standard reaction buffer at ~ 19 mg/mL protein and 3x molar excess of maleimide to protein. After 4 days of reaction at 4°C, more maleimide-activated HA-ADH was added to give a 9x molar excess over protein. After another 4 days reaction, more freshly prepared maleimide-HA was added to provide a final 13x molar excess. After 8 days of further reaction the sample was prepped for and run on FPLC.

The similar protocol with 177 mg derivatized HA, DM2-63, prepared as described in Example 1 and containing an estimated 37 µmoles hydrazide, was also performed with each protein.

Preparation of Protein Moieties

The preparation and purification of IL-1ra used in Example 2 of the present invention is set forth in published PCT Patent Publication No. WO 92/16221, hereby incorporated by reference including drawings.

The preparation and purification of the free-thiol osteoprotegerin (OPG) used in Example 2 of the present invention is set forth in published PCT Patent Publication No. WO 97/23614, hereby incorporated by reference including drawings.

The preparation and purification of the free-thiol (N-terminus) leptin used in Example 2 of the present invention is set forth in published PCT Patent Publication No. WO 96/05309, hereby incorporated by reference including drawings.

Claims

1. A hyaluronic acid-protein conjugate comprising at least one hyaluronic acid moiety attached to at least one protein moiety.

2. A hyaluronic acid-protein conjugate of Claim 1, wherein said conjugate exhibits increased bioefficacy as compared to protein alone.

3. A hyaluronic acid-protein conjugate produced by the method comprising:

(a) derivatizing a hyaluronic acid moiety to produce a derivatized hyaluronic acid;

(b) activating said derivatized hyaluronic acid moiety to produce an activated hyaluronic acid moiety;

(c) reacting said activated hyaluronic acid with a protein moiety to form a hyaluronic acid-protein conjugate;

(c) obtaining the hyaluronic acid-protein conjugate; and

(d) optionally, purifying the hyaluronic acid-protein conjugate.

4. A pharmaceutical composition comprising a hyaluronic acid-protein conjugate according to any of Claims 1 to 3 in a pharmaceutically acceptable carrier.